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RESEARCH ARTICLE

Chilling induces unidirectional solute leak through the locust gut epithelia

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ABSTRACT

Chill-susceptible insects, like the migratory locust, often die when exposed to low temperatures from an accumulation of tissue damage that is unrelated to freezing (chilling injury). Chilling injury is often associated with a loss of ion balance across the gut epithelia. It has recently been suggested that this imbalance is at least partly caused by a cold-induced disruption of epithelial barrier function. Here, we aimed to test this hypothesis in the migratory locust (*Locusta migratoria*). First, chill tolerance was quantified by exposing locusts to −2°C and recording chill coma recovery time and survival 24 h post-cold exposure. Longer exposure times significantly increased recovery time and caused injury and death. Ion-selective microelectrodes were also used to test for a loss of ion balance in the cold. We found a significant increase of haemolymph K^+ and decrease of haemolymph Na^+ concentration over time. Next, barrier failure along the gut was tested by monitoring the movement of an epithelial barrier marker (FITC-dextran) across the gut epithelia during exposure to −2°C. We found a significant increase in haemolymph FITC-dextran concentration over time in the cold when assayed in the mucosal to serosal direction. However, when tested in the serosal to mucosal direction, we saw minimal marker movement across the gut epithelia. This suggests that while cold-induced barrier disruption is present, it is apparently unidirectional. It is important to note that these data reveal only the phenomenon itself. The location of this leak as well as the underlying mechanisms remain unclear and require further investigation.

KEY WORDS: Chill tolerance, Chilling injury, Paracellular leak, Dextran, Alimentary canal

INTRODUCTION

Chill-susceptible insects are those that succumb to the negative effects of cooling at temperatures well above the freezing point of their extracellular fluids (Overgaard and MacMillan, 2017). When chilled, many ectotherms reach a temperature at which a state of neuromuscular paralysis (chill coma) occurs, known as chill coma onset temperature (CCO). Insects can remain in this reversible comatose state for the duration of a cold exposure in the lab (e.g. hours to days) and can often recover once the stressor (brief and/or mild cold) has been removed. The time taken to regain the ability to stand following chill coma is termed chill coma recovery time (CCRT) (Gibert et al., 2001; David et al., 1998). Both CCO and CCRT are regularly used as non-lethal means of quantifying chill susceptibility in various groups of insects, such as crickets (*Gryllus pennsylvanicus*), caterpillars (*Pringleophaga marioni*), fruit flies (*Drosophila melanogaster*), locusts (*Locusta migratoria*) and firebugs (*Pyrrhocoris apterus*) (Andersen et al., 2013, 2017a; Chown and Klok, 1997; Coello Alvarado et al., 2015; Robertson et al., 2017). In the event of a particularly harsh or prolonged exposure, an accumulation of cold-induced tissue damage (chilling injury) can occur (Koštál et al., 2006; MacMillan and Sinclair, 2011a). Chilling injury typically manifests as a loss of coordination, permanent limb paralysis or mortality (Overgaard and MacMillan, 2017). Quantifying an insect's condition or survival following cold stress is therefore another common measure of chill tolerance, and can be quantified using a scoring system (e.g. as dead/alive, or a range of conditions from dead to alive) to indirectly measure the degree of injury sustained (Findsen et al., 2013; Overgaard and MacMillan, 2017). While each of these chill tolerance traits operate via distinct mechanisms, they are all in some manner associated with a local or systemic loss of ion balance, suggesting that ionoregulatory failure may be a principal cause of chill susceptibility (Armstrong et al., 2012; Bayley et al., 2018; Findsen et al., 2013; Koštál et al., 2006; MacMillan et al., 2015a; Robertson et al., 2017).

In insects, organismal ion homeostasis is under tight regulation by both the Malpighian tubules (MTs) and gut epithelia (MacMillan and Sinclair, 2011a). Briefly, the gut has three main regions – the foregut, midgut and hindgut – each with its own specialized functions. The foregut lies at the anterior margin of the gut and consists of flattened and undifferentiated cells consistent with the lack of absorption or secretion within this region (Chapman, 2013). From the foregut, food travels to reach the remainder of the gut, where it is digested (Engel and Moran, 2013; Naikkhwah and O'Donnell, 2012). Cells along the midgut are actively involved in digestive enzyme production and secretion, acting as the primary site of digestion and nutrient, ion and water absorption (Chapman, 2013; Yerushalmi et al., 2018). Located at the posterior end of the midgut are the MTs, blind-ended tube-shaped diverticula that contain a multitude of cation exchangers and pumps (Maddrell and O'Donnell, 1992; O'Donnell and Ruiz-Sanchez, 2015). Through these ionoregulatory pumps (e.g. V-ATPase, the proton pump that primarily energizes transport at the MTs) and channels, ions such as K^+, Na^+ and Cl^- are driven from the haemolymph to the tubule lumen. These means of ion translocation across the MTs promote an osmotic gradient that favours the movement of water and unwanted waste products or toxins into the tubule lumen (secretion), producing primary urine (Maddrell and O'Donnell, 1992). Finally, the hindgut (composed of the ileum and rectum) is the most posterior region of the gut and is the main site for the absorption of water and solutes (Phillips et al., 1987). Cell membranes along the intercellular spaces of the hindgut are rich in Na^+/K^+-ATPase, which generates high $[Na^+]$ in the paracellular space that drives water across the hindgut epithelium via osmosis, permitting water reabsorption and production of a dry faeces (Des Marteaux et al., 2018; Phillips, 1981; Wall and Oschman, 1970).

Through the continuous ionoregulatory actions of the alimentary canal, the haemolymph of most insects contains high Na^+ and low K^+ concentrations at optimal or near-optimal thermal conditions

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(Engel and Moran, 2013; MacMillan et al., 2015b; Maddrell and O'Donnell, 1992). In cold conditions, however, the activity of ionoregulatory enzymes like V–ATPases and Na^+/K^+-ATPases is suppressed with a Q_{10} of approximately 3 (Bayley et al., 2018; Hosler et al., 2000; Mandel et al., 1980; McMullen and Storey, 2008; Moriyama and Nelson, 1989; Yerushalmi et al., 2018; MacMillan et al., 2015c). Over time, this temperature-induced suppression of transcellular ion transport often results in a net leak of haemolymph Na^+ and water (which follows Na^+ osmotically) to the gut lumen, effectively concentrating the K^+ that remains in the haemolymph (MacMillan and Sinclair, 2011b). Some intracellular K^+ simultaneously leaking down its concentration gradient into the extracellular space worsens this problem (Andersen et al., 2017a; MacMillan et al., 2014). As K^+ concentrations rise in the haemocoel (hyperkalaemia), the gradient of K^+ across the cell membrane is lost, and a marked depolarization in membrane potential occurs, eventually resulting in the activation of voltage-gated Ca^{2+} channels (Andersen et al., 2017a; Bayley et al., 2018; MacMillan et al., 2015a). The influx of Ca^{2+} that ensues is proposed to initiate a crippling cascade which causes a deterioration of cellular integrity, possibly through apoptosis (Mattson and Chan, 2003; Nicotera and Orrenius, 1998; Yi and Lee, 2011; Yi et al., 2007). Failure to maintain ion and water homeostasis in the cold can therefore ultimately result in organismal chilling injury or death. In turn, understanding the biochemical mechanisms underlying this failure may be essential to understanding chill susceptibility. While a cold-induced failure of transcellular transport is one likely mechanism of chilling injury and is under active investigation, ions do not cross epithelia solely via transcellular pathways (Donohoe et al., 2000; MacMillan et al., 2016a; O'Donnell and Maddrell, 1983).

In addition to the active movement of ions and passive transport of solutes and water through cells (transcellular pathways, e.g. via channels), the gut also relies on the passive movement, or leak, of molecules through paracellular pathways between adjacent cells (Jonusaite et al., 2016; le Skaer et al., 1987). Septate junctions are specialized cell–cell junctions analogous to vertebrate tight junctions that largely determine the permeability of these paracellular pathways (Jonusaite et al., 2016). Arthropod epithelia generally have two types of septate junctions: pleated and smooth. The former are typically observed in ectodermally derived epithelia such as the foregut and hindgut, and are 2–3 nm wide, while smooth septate junctions are found in endodermally derived tissues like the midgut, and are 5–20 nm wide (Jonusaite et al., 2016). To date, the majority of septate junction studies has been conducted on *Drosophila*, including the identification of septate junction types, associated proteins and septate junction influence in cold tolerance plasticity (Izumi and Furuse, 2014; Jonusaite et al., 2016; MacMillan et al., 2017). Septate junctions may be important to thermal plasticity; flies acclimated to colder conditions (6°C) are more tolerant of chilling than warm-acclimated flies (21.5°C) and upregulate approximately 60% of genes encoding known or putative fly septate junction proteins (MacMillan et al., 2016b). Cold-acclimated (10°C) flies also have reduced rates of paracellular leak of a fluorescent probe (FITC-dextran) from their gut lumen to their haemolymph, both before and during a cold stress, compared with warm-acclimated (25°C) flies (MacMillan et al., 2017). Together, these studies suggest that cold exposure can cause increased rates of leak through the paracellular barriers, and that improvements in cold tolerance may in part be related to an improved ability to maintain paracellular barriers. However, because flies were fed the probe for these experiments (and the gut was completely loaded with the probe upon cold exposure), the precise location of this leak along the gut, the mechanisms that drive it and whether this a problem experienced by all insects or just *D. melanogaster* remain unclear.

Here, we investigated the effects of chilling on epithelial barrier integrity in a chill-susceptible insect, the migratory locust (*Locusta migratoria*). As previously observed in *Drosophila*, we hypothesized that chilling disrupts septate junctions in the locust gut and that this effect leads to paracellular leak across the gut epithelia. Because of the often temperature–sensitive nature of ionoregulatory enzymes, and their dense concentration along ionomotive epithelia, we also hypothesized that this disruption in barrier integrity is limited to transport-rich segments along the locust gut such as the midgut and hindgut. To address these hypotheses, we first confirmed that our locust colony is chill susceptible by measuring their haemolymph ion content, survival and performance post-cold exposure. We then used the fluorescently labelled marker FITC-dextran to characterize directionality in cold-induced paracellular leak, and found that cold does induce paracellular leak through the gut epithelia of locusts, but surprisingly only in one direction.

MATERIALS AND METHODS

Experimental system

All experiments were conducted using male and female adult locusts, *Locusta migratoria* (Linnaeus 1758), aged 3–4 weeks post-final ecdysis. Locusts were obtained from a continuously breeding colony maintained at Carleton University in Ottawa, ON, Canada. This colony is reared under crowded conditions on a 16 h:8 h light: dark cycle at 30°C with 60% relative humidity (see Dawson et al., 2004). All animals had *ad libitum* access to a dry food mixture (oats, wheat bran, wheat germ and powdered milk), and fresh wheat clippings supplied 3 times per week.

CCRT and survival

The degree of chill susceptibility of the locust colony was quantified using both their CCRT and the degree of injury/mortality 24 h following exposure to −2°C. On the day of the experiment, locusts were collected from the colony, sexed by eye, and individually placed into 50 ml polypropylene tubes (n=10 per time point). These tubes were sealed using lids with small holes to allow access to air for the duration of the experiment. Excluding controls, all locusts were suspended using a Styrofoam rig in a cooling bath (Model AP28R-30, VWR International, Radnor, PA, USA) containing a circulating ethylene glycol:water mix (3:2) preset to 20°C and cooled to −2°C at a rate of −0.20°C min^{-1}. Both bath temperature and locust internal body temperature were monitored, the former via internal probes and the latter via inserted type-K thermocouples (TC-08 interface; PicoLog software version 5.25.3) located at the junction of the head and thorax of representative locusts (that were not used further in the experiments). Locusts were then left undisturbed at −2°C for 2, 6, 24 or 48 h, after which arbitrarily selected groups of locusts were removed from the cold and returned to room temperature (23°C). In order to monitor CCRT, insects were removed from their tubes and gently placed on the surface of a table and observed for the time taken to recover from chill coma. Animals were stimulated by gentle puffs of air from a transfer pipette every minute and were marked as having fully recovered when standing on all six limbs. Observation time was limited to 60 min; any locusts not meeting this criterion were marked as having not recovered.

After 60 min, these locusts were returned to their respective tubes along with a dry food mixture (oats, wheat bran, wheat germ and powdered milk) and water (supplied in microcentrifuge tubes with cotton) and left for 24 h at room temperature (23°C). An assessment of 24 h survival post-cold exposure was performed using a scoring

system of 0 to 5, similar to that described by MacMillan et al. (2014). Briefly, scores were defined as follows: 0, no movement observed (i.e. dead); 1, limb movement (slight leg and or head twitching); 2, greater limb movement, but unable to stand; 3, able to stand, but unable or unwilling to walk or jump; 4, able to stand, walk and/or jump, but lacks coordination; and 5, movement restored to pre-exposure levels of coordination.

Measuring paracellular leak in locusts

To measure paracellular permeability in the gut epithelia of locusts, we monitored the movement of a fluorescently labelled molecule in both the mucosal (lumen) to serosal (haemolymph) and the serosal to mucosal direction. All experiments used FITC-dextran (FD4; 3–5 kDa, Sigma-Aldrich, St Louis, MO, USA), a commonly used probe for determining paracellular permeability in both invertebrate and vertebrate models such as fruit flies (*D. melanogaster*), rats (*Rattus norvegicus domesticus*) and zebra fish (*Danio rerio*) (see MacMillan et al., 2017; Condette et al., 2014; Bagnat et al., 2007).

Quantification of mucosal to serosal paracellular leak

Haemolymph extractions were first used to measure leak across the gut epithelia in the mucosal to serosal direction. Here, locusts (n=12–14 per treatment time point; n=6 per control time point) were fed a mixture of dry food (oats, wheat bran, wheat germ and powdered milk) saturated with a solution of FITC-dextran in water (9.6×10^{-4} mol l^{-1}; selected based on standard curves from the preliminary trials) for 24 h prior to experiments. Pilot experiments confirmed the presence of FITC-dextran throughout the alimentary canal the following day and that FITC-dextran ingestion did not affect survival (survival for control locusts with and without FITC-dextran injection was scored as 5). Similar to the protocol for CCRT (outlined above), animals were suspended in a cooling bath preset to 20°C and cooled to −2°C at a rate of −0.2°C min^{-1}. Insects were then held at −2°C (or room temperature for controls) and left undisturbed for 2, 6, 24 or 48 h. Upon removal of the locusts from the cold, haemolymph samples were collected using methods adapted from Findsen et al. (2013). Briefly, locusts were pricked dorsally using a dissecting probe at the junction of the head and thorax before using a 50 µl capillary tube to collect an excess of haemolymph. A 2 µl aliquot of haemolymph was pipetted into 96-well plates (Corning Falcon Imaging Microplate; black/clear bottom) and diluted 50-fold with locust saline (in mmol l^{-1}: 140 NaCl, 8 KCl, 2.3 $CaCl_2$ dihydrate, 0.93 $MgCl_2$ hexahydrate, 1 NaH_2PO_4, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 Hepes, pH 7.2). Samples were then analysed for FITC-dextran content via fluorescence spectrophotometry (excitation: 485 nm, emission: 528 nm; BioTek Cytation 5 Imaging Reader, Winooski, VT, USA). Pilot experiments showed no interference from the saline when measuring fluorescence. Lastly, concentrations of FITC-dextran in the samples was determined by reference to a standard curve of FITC-dextran in locust saline.

Quantification of serosal to mucosal paracellular leak

With leak confirmed in the mucosal to serosal direction (see Results), experiments were conducted in the serosal to mucosal direction (from the haemolymph to the gut lumen). This was done both to corroborate the presence of leak across the gut epithelia and to isolate the area across which leak occurred. Protocols for this novel leak assay were developed through preliminary trials. In the final assay, FITC-dextran was dissolved in locust saline, resulting in a final FITC-dextran concentration of 3.84×10^{-3} mol l^{-1} (selected based on standard curves from the preliminary trials). Using a 25 µl Hamilton syringe, 20 µl of this solution was injected into the haemocoel ventrally at the junction of the thorax and first abdominal segment of locusts. Pilot experiments revealed that neither the 20 µl injection nor the FITC-dextran itself impacted locust performance or survival (survival for control locusts with and without FITC-dextran injection was scored as 5). As with the previous experiments, animals were exposed to −2°C (or room temperature for controls) for 2, 6, 24 or 48 h (n=4–6 locusts per time point).

Locusts were individually removed from the cooling bath and dissected within 15 min of their target cold exposure duration for tissue collection. Each dissection was completed within 7 min. Animals were killed by decapitation before removing all limbs and wings. The thorax and abdomen (containing the internal organs) were placed in a Petri dish lined with silicone elastomer (Sylgaard 184 Silicone Elastomer Kit, Dow Chemical, Midland, MI, USA) and containing locust saline. A longitudinal incision was made in the anterior to posterior direction along the ventral side to expose the gut. With the body wall pinned back, the tracheae and MTs were then cleared away to access the gut tissue. The gut was then cut into three segments (anterior, central, posterior) based on our ability to carefully isolate these segments rather than pre-determined anatomical divisions (see Fig. 3A). Briefly, the anterior segment was defined as the foregut to the anterior midgut caeca, the central segment as the posterior midgut caeca to the midgut–hindgut junction, and the posterior segment as the midgut–hindgut junction to the rectum. In an effort to account for individual variation, all three gut segments were dissected from the same locust. Additionally, excessive leak of gut contents during collection was mitigated by gently pinching segments with dissecting forceps at both ends before excision. Upon removal, segments were washed briefly in saline (while retaining their contents) to remove any excess dextran-saturated haemolymph, and placed in microcentrifuge tubes containing 500 µl of locust saline. Samples were subsequently homogenized (OMNI International Tissue Master 125 120 V, Kennesaw, GA, USA; approximately 3 min), sonicated (Qsonica Sonicators Model CL-188, Newtown, CT, USA; 3×5 s bursts with 10 s rests), and centrifuged for 5 min at 10,000 ***g***. A 100 µl aliquot of the resulting supernatant was collected and transferred to a black 96-well plate for fluorescence spectrophotometry. Control samples confirmed that tissues from locusts that were not injected with the probe had negligible fluorescence (see Fig. 3B,D).

Because little FITC-dextran appeared in the gut samples (see Results), haemolymph extraction experiments were performed on separate locusts over identical exposures to determine how much FITC-dextran was being lost from the haemolymph over time. Similar to the above protocols, a new set of locusts (n=4–6 locusts per time point) were injected with the FITC-dextran solution and suspended at −2°C for 2, 6, 24 or 48 h in a circulating cooling bath, while controls were held at room temperature. After the designated exposures, haemolymph samples were collected in excess via 50 µl capillary tube and analysed using the aforementioned methods.

Cold-induced loss of ion balance

To maximize use of the large volumes of available haemolymph, locusts used for the FITC-dextran haemolymph extractions (above) were also used to collect data on haemolymph ion balance over time. Using the haemolymph collected in excess via 50 µl capillary tubes, 10 µl of haemolymph from each animal was collected (K^+: n=5–6 locusts per time point; Na^+: n=4–6 locusts per time point). These 10 µl samples were promptly vortexed and flash frozen in liquid nitrogen to avoid coagulation of the haemolymph, and stored at −80°C until experiments. All samples were vortexed once again prior to testing. Haemolymph Na^+ and K^+ concentrations were measured using

custom-made ion-selective microelectrodes following previously described methods (Jonusaite et al., 2011). No interference from the FITC-dextran was found when measuring ions. The ion content of haemolymph both with and without FITC-dextran was measured in control (room temperature) locusts over 48 h, and statistical analysis revealed no significant differences in ion concentrations between the two groups (linear model, $F_{1,10}$=0.302, P=0.594). Microelectrodes were constructed from thin-walled, borosilicate glass capillaries (item number TWI50-4, World Precision Instruments, Sarasota, FL, USA) pulled to a tip diameter of ∼3 µm using a pipette puller (P-1000, Sutter Instruments, Novato, CA, USA). Our Na^+–selective microelectrodes were created using 100 mmol l^{-1} NaCl backfill solution and Na^+ ionophore II cocktail A (Sigma-Aldrich), while K^+-selective microelectrodes contained 100 mmol l^{-1} KCl backfill solution and K^+ ionophore I cocktail B (Sigma-Aldrich). The circuit was completed using a pulled filamented capillary containing 500 mmol l^{-1} KCl. Microelectrodes were calibrated using 10 and 100 mmol l^{-1} NaCl or KCl standards (osmolality adjusted with LiCl) for their respective measurement. These standards were also used to calculate the ion concentration in samples of haemolymph from the obtained voltage measurements using the following equation:

$$C_f = C_L\ 10^{\Delta V/S}, \quad (1)$$

where C_f is the final concentration (in mmol l^{-1}), C_L is the concentration (in mmol l^{-1}) of the lowest standard used for the data point of interest, ΔV is the difference (in mV) between the sample of interest and the lowest standard, and S is the slope (the difference in mV between the two standards). Only microelectrodes with a slope between 50 and 60 mV (close to the expected Nernst slope of 58 mV) were used for all experiments (mean electrode slopes: Na^+=51.2±0.1 mV; K^+=55.5±0.4 mV).

Data analysis and availability

All data, excluding concentrations of FITC-dextran found in the haemolymph (*in vivo* FITC-dextran fed experiments; outlined below), were analysed using general linear models in R Studio version 1.2.1335 (https://www.rstudio.com) and R version 3.0.2. In addition to glms, the effects of time in the cold on gut leakiness (quantified by FITC-dextran movement into the gut) were also analysed using a linear mixed effects model via the lmer() function in R (lme4 and lmerTest packages for R). Time and segment were treated as fixed effects, while locust ID (*in vivo* FITC-dextran injection experiments) was treated as a random effect to account for variability in locust gut leakiness per individual. The effects of time in the cold on gut leakiness (quantified here by FITC-dextran movement into the haemolymph) were analysed using a linear model (two-way ANOVA) in R. All data were analysed with time as both a continuous and categorical factor; however, the outcomes of these two approaches were identical. As such, all results presented treat time as a continuous factor. Locust sex was initially included in our statistical models (as a fixed effect) and was excluded if non-significant. Where sex significantly impacted a variable, it is reported in the results below. Because of an error, sex was not recorded for each individual in our feeding experiment, although sexes were intentionally balanced. The level of statistical significance was 0.05 for all analyses, while all values presented are means±s.e.m. All data are available in Table S1.

RESULTS

CCRT and injury following chilling

The chill susceptibility of our locust colony was confirmed by measuring CCRT and scoring injury/survival of locusts 24 h post-cold exposure. After 2 h of cold exposure, all animals recovered from chill coma to standing position within 10–18 min. Expectedly, recovery time significantly increased as exposure time grew longer (Fig. 1A; linear model, $F_{1,22}$=42.3, P<0.001). This trend persisted until the last time point (48 h at −2°C), at which point no locusts recovered the ability to stand within 60 min. Similarly, survival rates decreased with longer cold exposures, leading to nearly 100% mortality after 48 h at −2°C (n=4 locusts remained technically alive, but demonstrated low functionality; see Fig. 1B; linear model, $F_{1,38}$=188, P<0.001). Finally, while significant effects of locust sex were found on CCRT (see Fig. S1 for breakdown by sex; linear model, $F_{1,21}$=7.06, P=0.015), there was no significant interaction between sex and time exposed to −2°C (linear model, $F_{1,20}$=2.71, P=0.115).

Haemolymph ion concentrations

Ion-selective microelectrodes were used to determine the effects of cold exposure on extracellular ion balance over the course of our experiments. While the concentration of Na^+ in the haemolymph decreased significantly as time at −2°C increased, up until approximately 24 h (Fig. 1D; linear model, $F_{1,23}$=10.9, P=0.003), haemolymph K^+ concentration significantly increased, doubling from 11.8 mmol l^{-1} to 23.3 mmol l^{-1} over 48 h at −2°C (Fig. 1C; linear model, $F_{1,23}$=34.2, P<0.001). Neither Na^+ nor K^+ concentration in the haemolymph was directly impacted by sex (see Fig. S1 for breakdown by sex; linear model, $F_{1,22}$=3.55, P=0.073; linear model, $F_{1,22}$=2.96, P=0.100, respectively). There was, however, a marginally significant interactive effect of time spent at −2°C and locust sex on haemolymph [K^+]; female locusts had a tendency to suffer from slightly more severe hyperkalaemia in the cold (Fig. S1; linear model, $F_{1,21}$=4.34, P=0.050).

Mucosal to serosal leak

We first examined cold-induced leak in the mucosal to serosal direction. Locusts were fed a dry food mixture saturated with water containing a fixed concentration of FITC-dextran and sampled for marker content. These experiments yielded a significant and near-linear increase in haemolymph FITC-dextran concentration over time in the cold (Fig. 2; linear model, $F_{1,46}$=7.99, P=0.007). Finally, significant differences were also observed between treatment groups (Linear model, $F_{1,44}$=9.08, P=0.004). There was approximately a 21-fold difference in haemolymph FITC-dextran levels between control locusts and those that spent 48 h at −2°C.

Serosal to mucosal leak

Traditionally, studies examining paracellular leak of FITC-dextran and other large markers like inulin involve the oral administration of probes to the animals (as above). With the presence of mucosal to serosal leak confirmed, we quantified the presence of cold-induced paracellular leak across the gut epithelia in the opposite direction (i.e. from the haemocoel into the gut). To do so, samples were taken from each gut segment (Fig. 3A; anterior, central and posterior) and analysed for fluorescent content following marker injection. Analysis of these data treated locust sex as a random effect to account for variability in locust gut leakiness per sex or individual. Interestingly, while FITC-dextran concentration did significantly increase in the gut over time (Fig. 3B; Linear model, $F_{1,82}$=7.46, P=0.008), less than 1% of the total injected marker appeared within the gut after 48 h in the cold. Using summary data from both the gut leak assay (0.019±0.003 mg ml^{-1}) and haemolymph extraction experiments (see below; 2.05±0.279 mg ml^{-1}), we estimate that approximately 0.93% of total FITC-dextran injected into the haemolymph leaked across the gut

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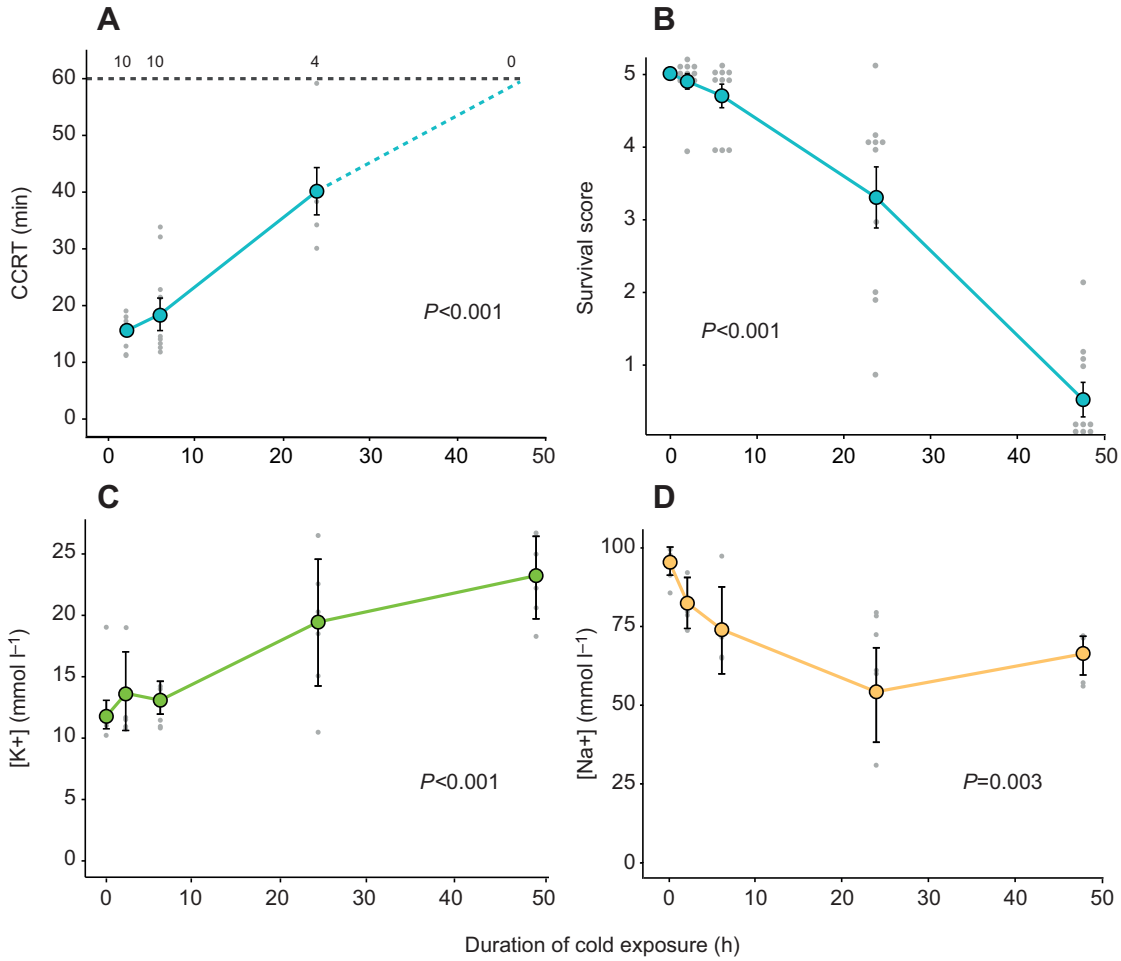

Fig. 1. *Locusta migratoria* suffer from injury and ionoregulatory collapse typical of chill-susceptible insects. (A) Chill coma recovery time (CCRT) of locusts (mixed sexes; n=10 per time point) held at −2°C for 2, 6, 24 or 48 h. Locusts were observed for 60 min following cold exposure and were marked as having recovered when standing on all six limbs. Values above the dashed black line represent the number of locusts that had recovered within 60 min. The solid blue line represents mean values per time point. (B) Locust condition (survival) following exposure to −2°C for 0, 2, 6, 24 or 48 h (n=10 per time point). Survival score was based on the following: 0, no movement observed (i.e. dead); 1, limb movement (leg and or head twitching); 2, moving, but unable to stand; 3, able to stand, but unable or unwilling to walk or jump; 4, able to stand, walk and/or jump, but lacks coordination; and 5, movement restored to pre-exposure levels of coordination. The solid blue line represents mean values per time point. To show all data points, dots are clustered around their respective score (where applicable). (C) Changes in locust haemolymph K^+ concentration over time spent at −2°C (n=5–6 locusts per time point). (D) Changes in locust haemolymph Na^+ concentration over time spent at −2°C (n=4–6 locusts per time point). Values are means±s.e. Light grey dots represent each sample taken per time point. Error bars not shown are obscured by the symbols.

epithelia into the lumen during the entire 48 h cold exposure. This method made it possible for us to isolate potential sites of barrier loss along the gut. However, there were no significant differences in marker concentration among the three gut segments (linear model, $F_{2,80}$=2.46, P=0.093). Similarly, no significant interaction was found between the time spent at −2°C and the segment type (linear model, $F_{2,77}$=1.50, P=0.231). Interestingly, sex significantly impacted FITC-dextran concentration within the gut (see Fig. S2A–C; linear model, $F_{1,79}$=5.41, P=0.023), but sex did not significantly interact with time spent in the cold or gut segment used (linear model, $F_{1,72}$=0.060, P=0.942). Finally, total concentration of FITC–dextran found in the gut did not significantly differ between treatment (−2°C) and control locusts (23°C) at 24 h (Fig. 3C; linear model, $F_{4,1}$=0.531, P=0.758). There was, however, a significant difference between the total concentration of FITC–dextran sampled at 0 h and that sampled at 24 h in both temperature treatments, where locusts treated for 24 h had significantly higher concentrations of FITC-dextran within their whole guts (linear model, $F_{1,11}$=25.4, P<0.001).

Loss of marker from the haemolymph was also investigated to corroborate levels of cold-induced leak into the gut and to determine whether our locusts were capable of metabolizing the probe. Samples taken at time 0 h (ranging from 1.23 to 2.84 mg ml^{-1} probably in response to locust size) align with the average estimated haemolymph FITC-dextran concentration of 1.59 mg ml^{-1} (haemolymph volume approximated using values obtained by Ayali and Pener, 1992). Over 48 h, however, there was a significant loss of FITC–dextran from the haemolymph at both −2°C and 23°C (Fig. 3D; linear model, $F_{1,41}$=8.82, P=0.005), although no significant difference in marker movement between the two treatment groups was found (linear model, $F_{1,40}$=0.239, P=0.628). We also observed no significant interaction between marker concentration and time in the −2°C and 23°C conditions (linear model, $F_{1,38}$=0.147, P=0.704). As with the amount of FITC-dextran found within the gut, locust sex had a significant effect on haemolymph FITC-dextran concentration (Fig. S2D; linear model, $F_{1,39}$=29.4, P<0.001) but did not interact significantly with time spent at either −2°C or 23°C (linear model, $F_{1,35}$=0.145, P=0.706).

DISCUSSION

Chill-susceptible insects experience adverse effects of chilling at low temperatures that occur in the absence of ice formation. The

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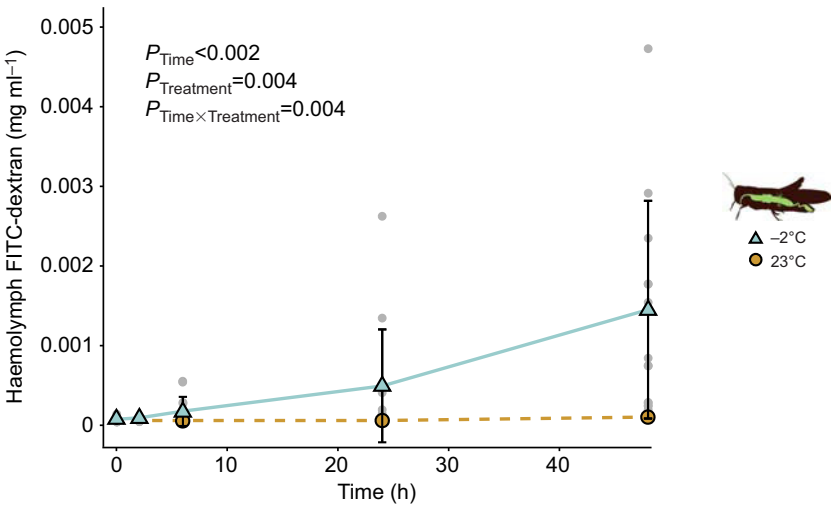

Fig. 2. FITC-dextran leaks in the mucosal to serosal direction across the gut of *L. migratoria*. Levels of haemolymph FITC-dextran following oral administration of the marker and 2, 6, 24 or 48 h at –2°C (n=12–14 per time point). The dashed line represents control locusts sampled across 48 h at 23°C (n=6 per time point). Values are means ±s.e. Grey points represent each sample taken per time point. Error bars not shown are obscured by the symbols.

consequences of cold exposure for these insects, like chill coma and tissue damage, are consistently associated with a disruption of ion and water balance (Overgaard and MacMillan, 2017). Although temperature effects on active ion transport processes are likely to be critical drivers of organismal failure, another potential contributor is cold-induced deterioration of paracellular barrier components known as septate junctions. In this study, we provide the first evidence for the presence of unidirectional cold-induced paracellular leak. To our knowledge, this is also the first evidence of paracellular leak in cold-exposed animals other than *Drosophila*. Our findings provide additional correlative evidence of a role for epithelial barrier function as a contributing factor in insect chill tolerance.

After the chill susceptibility of our locusts was verified (Fig. 1; Fig. S1), the effect of cold on paracellular leak across the gut epithelia was investigated. To do so, we first fed locusts a dry food–FITC-dextran mixture 24 h prior to cold exposure. Similar to trends observed in *Drosophila* using a similar approach, we predicted that a large and rapid increase of FITC-dextran concentration in the locust haemocoel (i.e. mucosal to serosal leak) would occur over time in the cold (MacMillan et al., 2017). Accordingly, we observed a significant increase in haemolymph FITC-dextran concentration over time in locusts at −2°C, resulting in a nearly 8- and 23-fold rise in total FITC-dextran levels after 24 h and 48 h in the cold, respectively (Fig. 2). Interestingly, the former increase of FITC-dextran under cold stress was similar to that seen across 24 h in FITC-dextran-fed *Drosophila*, where a 10.5–fold increase in marker concentration was observed (MacMillan et al., 2017). In addition to *Drosophila*, studies spanning an array of models from kissing bugs (*Rhodnius prolixus*) to mice (*Mus musculus*) and killifish (*Fundulus heteroclitus*) have also documented the movement of various markers across gut epithelia through the paracellular pathways in the mucosal to serosal direction (Andersen et al., 2017b; le Skaer et al., 1987; MacMillan et al., 2017; O'Donnell and Maddrell, 1983; O'Donnell et al., 1984; Wood and Grosell, 2012; Woting and Blaut, 2018).

To isolate areas of this cold-induced leak across the gut epithelia, experiments were next conducted in the opposite direction through FITC-dextran injection (see Fig. 3A for gut segment breakdown). However, when each gut segment was analysed for marker content, we found that cold stress induced minimal leak from the haemolymph into the gut (Fig. 3B). Even more striking was the lack of difference found between control and cold-exposed locusts when the total amount of FITC-dextran within the gut at 24 h was compared (Fig. 3C). These results therefore suggest that a slight leak occurs in the serosal to mucosal direction, though it is not temperature sensitive. Similarly, we also observed no difference between the amount of FITC–dextran in the haemolymph in the cold and at room temperature (Fig. 3D). It is important to note that while we saw significant effects of sex on the concentration of injected FITC-dextran in the gut and remaining in the haemolymph (Fig. S2), these results are probably due to size discrepancies between male and female locusts. This difference between the two sexes is most readily seen in our haemolymph extractions (Fig. S2D), where the larger females have significantly lower FITC-dextran concentrations in their haemolymph than the smaller males. These differences in size, however, did not affect the general lack of a main effect of cold exposure. Finally, although we observed decreasing levels of FITC-dextran from the haemolymph overall, the majority of the injected marker was retained in the haemocoel after a prolonged period of time. Such FITC-dextran retention within the extracellular space (whether through a lack or minimal presence of marker degradation) has also been observed in vertebrate models like rats (*R. norvegicus domestica*), as well as in invertebrate models like the plant bug *Lygus hesperus*, lepidopterans (*Malacosoma disstria*, *Manduca sexta*, *Orgyia leucostigma* and *Orgyia pseudotsugata*) and orthopterans (*Schistocerca americana*, *Melanoplus sanguinipes* and *Phoetaliotes nebrascensis*; Barbehenn and Martin, 1995; Habibi et al., 2002; Nejdfors et al., 2000). Based on this evidence, we excluded FITC-dextran metabolism or excretion as a plausible explanation for our nominal marker movement and remain confident that dextran is a good marker of paracellular permeability when injected into the haemocoel.

The degree of marker movement (or lack therefore) that we observed in the serosal to mucosal direction is very small when compared with results in fed *Drosophila*, where even cold-acclimated (and more cold-tolerant) flies exhibited 10.5-fold increases in haemolymph FITC-dextran levels in the cold (Andersen et al., 2017b; MacMillan et al., 2017). By contrast, leak across the gut of our locusts into the gut lumen resulted in only a 2–fold increase in the cold relative to concentrations in animals prior to cold exposure (Fig. 3B). In contrast, other macromolecules at lesser concentrations have been shown to leak across the gut, albeit from the gut into the extracellular compartment. The gut of fifth instar desert locusts (*Schistocerca gregaria*) and the midgut of *Aedes aegypti* (yellow fever mosquito), for instance, are permeable to inulin (~5 kDa) and FITC-dextrans as large as 148 kDa, respectively (Zhu et al., 2001; Edwards and Jacobs-Lorena, 2000). This permeability to large molecules is not a

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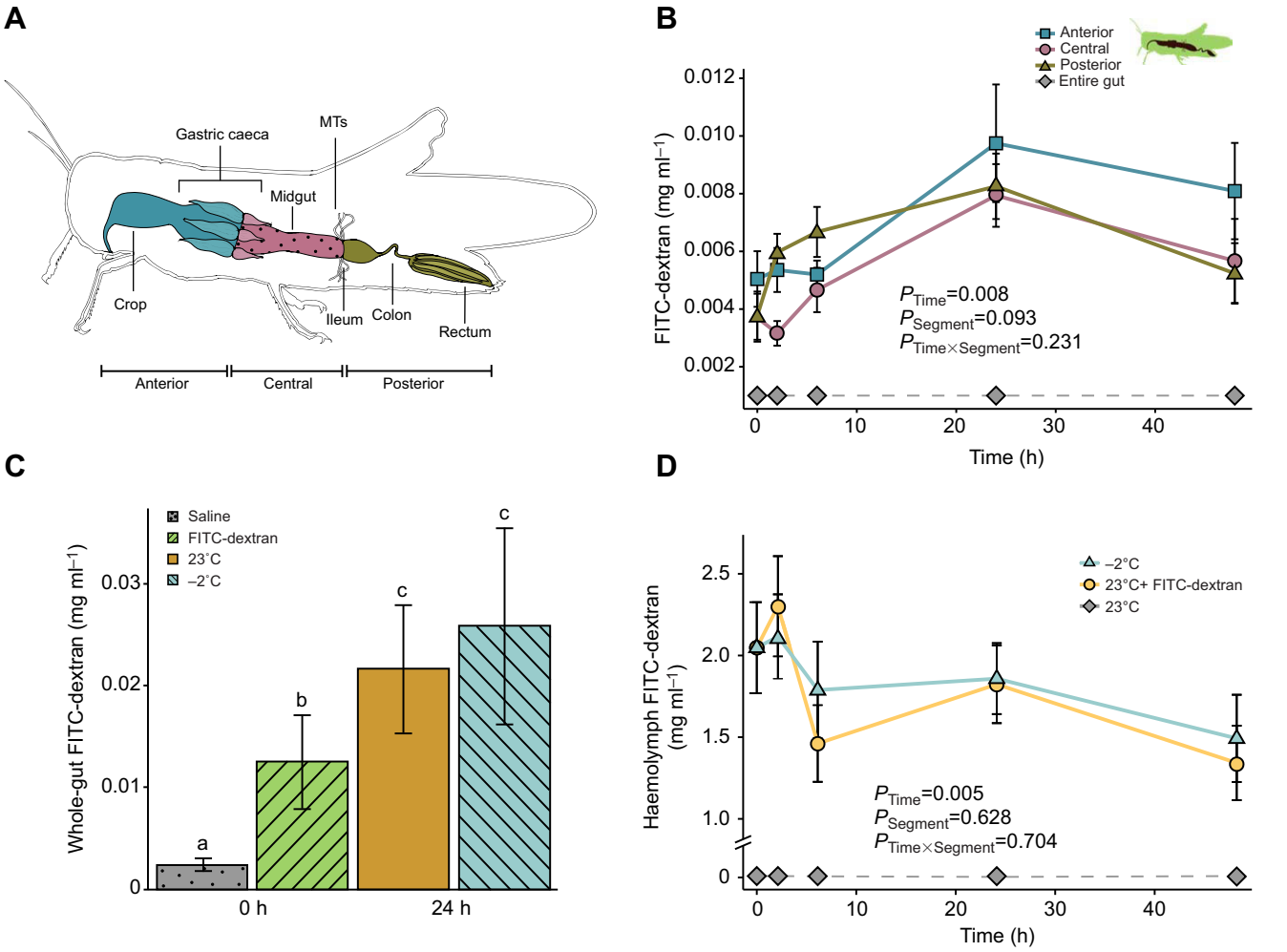

Fig. 3. Cold stress causes minimal leak of FITC-dextran across the gut epithelia of *L. migratoria* in the serosal to mucosal direction. (A) A schematic diagram of the locust gut tract sectioned into three segments (anterior, central and posterior). Relative to the locust gut anatomy, the segments were determined as follows: anterior – foregut to the anterior midgut caeca; central – posterior midgut caeca to the Malpighian tubules (MTs; removed; the midgut–hindgut junction); posterior – MTs to the rectum. Figure illustrated from observation. (B) Concentration of injected FITC-dextran present in each gut segment (anterior, central and posterior; n=4–6 per group) after exposure to –2°C for 2, 6, 24 or 48 h. Entire gut values are for the no FITC-dextran control. (C) Mean values representing the total FITC-dextran content within the gut (sum of anterior, central and posterior segments) at 24 h of exposure to either 23°C (control) or –2°C (n=4–6 per group). Means shown at 0 h were sampled immediately post-injection (FITC-dextran or saline). Letters denote a significant difference (P_{Time}<0.001; $P_{Treatment}$=0.758). (D) Concentration of injected FITC-dextran remaining in the haemolymph over 48 h at –2°C and at 23°C (n=4–6 per group). Control flies were held at 23°C with no FITC-dextran injection. Values are means±s.e. Error bars not shown are obscured by the symbols.

phenomenon limited to invertebrates. On the contrary, numerous intestinal permeability experiments have been done in vertebrate models such as mice and rats using FITC-dextran – the vast majority of which support the ability of FITC-dextran to diffuse across areas of the gut (Pantzar et al., 1993; Woting and Blaut, 2018). While our findings clearly differ from those previously reported, they are consistent with the inability for FITC-dextran to permeate the locust rectal wall (Gerber and Overgaard, 2018). In this case, however, gut preparations were exposed to a short-term cold stress. Exposure to more prolonged bouts of cold may yield different results. Altogether, these data suggest that cold-induced leak does occur in locusts, but occurs unidirectionally across the gut epithelia during cold stress.

It is well documented that cold exposure causes not only a loss of ion and water balance but also a depolymerization of cytoskeletal components such as actin (Belous, 1992; Callaini et al., 1991; Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009; Kim et al., 2006). The actin cytoskeleton is critical to ion transport regulation, often acting as an anchor point to which transport proteins attach on both the basolateral and apical borders of epithelial cells (Cantiello, 1995a; Cantiello et al., 1991; Janmey, 1998; O'Donnell, 2017; Sasaki et al., 2014). Damage to the actin cytoskeleton can therefore create a cascade of detrimental effects within an organism. For instance, disruptions in the actin cytoskeleton have been shown to inactivate K^+ channels in human melanoma cells (Cantiello et al., 1993). Similarly, in rat kidneys, failure of the cytoskeletal system stimulates Na^+/K^+-ATPase activity such that it has an increased affinity for Na^+ (Cantiello, 1995b). Such a disruption of ionoregulation could in turn directly compromise water and Na^+ secretion and/or reabsorption within insects – especially in the cold.

In addition to its role in regulating ion transport, actin has also been linked to the maintenance of tissue integrity as a key component of epithelial structure (Lane and Flores, 1988; Woods and Bryant, 1991). As septate junctions are typically located on the apical borders of epithelial cells, cold-induced disruption in septate junctions, as seen with *Drosophila* (MacMillan et al., 2017), may be caused by cold-induced disassembly of the cytoskeletal network (Belous, 1992;

Harvey and Zerahn, 1972). Coupled with a failure of ion transporters and channels on the apical borders, such a loss of tissue integrity could lead to a functionally 'funnel-like' cavity along the mucosal side of gut epithelia. This further deterioration of barrier integrity in the cold may exacerbate the damage and leak of gut contents into the haemocoel and contribute to the cascade of events which result in the damage and death typically seen in cold-exposed insects. It is important to note that the surface of gut epithelial cells may differ in transport and septate junction properties, potentially resulting in only a section or sections along the gut being vulnerable to cold-induced structural damage (Cioffi, 1984; Harvey and Zerahn, 1972). Nevertheless, such structural deterioration along the mucosal side of the gut epithelia may account for the fact that FITC-dextran, despite its large and bulky composition, is able to move from the gut lumen into the haemocoel, but not in the opposite direction.

In conclusion, locusts, like *D. melanogaster*, experience cold-induced leak from the gut when exposed to low temperatures. It remains entirely unclear whether this unidirectional leak occurs under other conditions of stress and in other animal models, but in locusts (and possibly other insects), this leak across the gut may be attributed to a cold-induced deterioration of paracellular barrier integrity along the mucosal surface of the gut. While this study presents evidence supporting a directionality of cold-induced leak along the gut, the precise location of this leak and the mechanisms that drive it, unfortunately, remain unclear. To work toward filling these knowledge gaps, we propose further investigation of the effects of cold exposure on the locust gut be conducted in isolated *ex vivo* gut sac preparations (Hanrahan et al., 1984). Such an experimental setup would allow each gut region to be more carefully assayed for the presence of cold-induced damage and/or leak. We found that there is the wide variability in the degree of paracellular barrier failure among individual cold-exposed locusts (Fig. 2). If barrier failure is central to the progression of chilling injury, these differences in individual leak rates may closely reflect survival outcomes (Fig. 1B). Combining methods of assessing survival and FITC-dextran movement (i.e. haemolymph extractions) may therefore yield useful information regarding this individual variation, and perhaps a new method for measuring and explaining insect chill susceptibility.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.B., H.M.; Methodology: K.B., H.M.; Resources: H.M.; Data curation: K.B.; Writing - original draft: K.B.; Writing - review & editing: H.M.; Visualization: K.B.; Supervision: H.M.; Project administration: H.M.; Funding acquisition: H.M.

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